BARD Final Report US-4700-14

Transfected *Babesia bovis* expressing the anti-tick Bm86 antigen as a vaccine to limit tick infestation and protect against virulent challenge

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Three year research project

Abstract

Bovine babesiosis, caused by the apicomplexan parasites *Babesia bovis* and *B. bigemina*, is a major tick borne disease of cattle with significant economic importance globally. The vectors of Babesia parasites are *R. (Boophilus) annulatus* and *R. microplus*. In Israel these parasites are transmitted manly by *R. annulatus*. The main goal of the proposal was developing and testing a novel *B. bovis* vaccine based on stably transfected attenuated *B. bovis* expressing the anti-tick Bm86 antigen. This required generating a transfected-attenuated *B. bovis* parasite containing a bidirectional promoter expressing both, the gfp-bsd selectable marker and the tick vaccine antigen Bm86. The vaccine was tested for its ability to elicit protective immune responses against *T. annulatus* ticks. Efficient control of babesiosis is based on a complex scheme of integrated management, including preventive immunization, anti-babesial chemotherapy and control of tick populations. Live vaccines based on attenuated parasites are the most effective measure to control babesiosis, and are currently used in several countries, including Israel. Live attenuated parasites lead to a chronic infection and development of strong and long term immunity in vaccinated cattle. Still, live vaccines have several limitations, including the difficulty to distinguish among vaccinated and naturally infected cattle and potential for sporadic outbreaks in vaccinated animals. Tick limitation is essential to control babesiosis but the main measure to reduce tick infestation is traditionally approached using acaricides, which is limited by environmental concerns and the development of resistance by the ticks. Alternative tick-control measures including the use of anti-tick vaccines are emerging, and at least partial protective immunity has been achieved against tick vectors by vaccination with recombinant protective tick antigens (ie: Bm86). In addition, the Babesia vaccine development toolbox has been recently expanded with the development of transfection technology in Babesia parasites. In this approved proposal we successfully developed a Babesia live attenuated transfected vaccine, which is able to express a *B. bovis* MSA-1 signal-Bm86 chimera and eGFP genes under the control of the B. bovis ef-1α and actin promoters respectively. Genetic analysis demonstrated specific stable integration of the transfected genes in the expected ef-1α locus, and immunofluorescence analysis confirmed expression of Bm86 in the surface of transfected parasites. When applied to splenectomized calves, the transfected parasites were able to cause persistent *B. bovis* infection with production of antibodies reactive with Bm86 for at least six months. In addition, partial protection against ticks was also observed upon challenging the vaccinated animals with *R. annulatus* larvae. However, when used on intact calves, the vaccine failed to elicit detectable immune responses against Bm86, and we are still in the process of interpreting the data and make necessary changes in our experimental approaches. Overall, the results obtained here represent a step forward towards the development of integrated vaccines against both ticks and tick –borne pathogens, using the Babesia attenuated parasites as a platform to the delivery of exogenous protective antigens

Summary Sheet

Publication Summary

PubType	IS only	Joint	US only
Review Article	0	2	0
Reviewed	0	0	1

Training Summary

Trainee Type	Last Name	First Name	Institution	Country
Postdoctoral Fellow	Levi	Maayan	Kimron Veterinary Institute	Israel
Ph.D. Student	Kelinerman	Gabriela	KVI	Israel

Contribution of Collaboration:

Two studies visits, one each for both US and Israel researchers, were performed during the project. The two research groups interacted on a permanent basis to mutually report and discuss the ongoing work. Experimental protocols were exchanged and jointly analyzed, and the planned work was carefully discussed among the US and Israel groups before experiments were performed. The US and the Israel PI actively engaged in discussion on the pitfalls and direction of the research using Skype and other similar social media on a regular basis. In addition, the two research groups collaborated in the development of a stable *B. bigemina* transfection system. The work was submitted for publication to the Journal Scientific Reports for review. Importantly, this new transfection system expands the possibilities for expression of vaccine tick antigens in other important *Babesia* species, which is a usual component of the current live vaccine. The transfection and Bm86 expression work were performed in the US lab whereas the tick transmission, the animal experiments and quantitative PCR research were performed in the Israel labs.

Achievements and potential impacts:

The main achievements of the financed proposal included:

1. Generation of a transfected parasite line able to express both gfp-bsd and the tick protective antigen Bm86. (Appendix, 1.)
2. Expression of the vaccine Bm86 antigen in the surface of transfected parasites for increased antigenicity. (Appendix, 1.)
3. Development of stable transfection system for *B. bigemina* that will expand the potential of transfected vaccines. (Appendix, 2.)
4. Confirmation of the lack of transmissibility of the *B. bovis* Israeli vaccine strain by *R. annulatus* ticks. (Appendix, 3.)
5. Development of a quantitative real time PCR (Appendix 4.)
6. Vaccination of bovines with the transfected parasite line caused mild disease and persistent infection with long term production of antibodies against Bm86 (Appendix 4).
7. Challenge of vaccinated with *R. annulatus* ticks resulted in reduced tick load in vaccinated animals compared to a non-vaccinated control (Appendix 6.).

Potential impact of the outcomes in Agriculture

The results of the experiments support further development of a dual vaccine aimed at control both bovine babesiosis and tick load in bovines Availability of more efficient dual anti-Babesia and tick vaccines will impact animal health, profitability of farmers, increased red meat production, and it will protect the environment by diminishing the use and negative effects of effect of acaricides.

Changes of original research Plan:

A nine month extension of the BARD project was requested and approved in order to proceed with animal vaccination involving a larger number of animals and challenge experiments in order to demonstrate the efficiency of the vaccine developed in this Project.

Publications for Project US-4700-14

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun
Published	Reviewed	*Silva, MG, Knowles, D., Suarez CE*	Identification of interchangeable cross-species function of elongation factor-1 alpha promoters in Babesia bigemina and Babesia bovis	***Parasites and Vectors***	: 2016	US only
Published	Review Article	*Maayan Margalit Levi, Sharon Tirosh-Levy, Roee Dahan, Dalia Berlin, Amir Steinman, Nir Edery, Igor Savitski, Benjamin Lebovich, Don Knowles, Carlos E. Suarez, Gad Baneth, Monica L. Mazuz*	First Detection of Diffuse and Cerebral Theileria equi Infection in Neonatal Filly	***Journal of Equine Veterinary Science***	60 : 23-28 2018	Joint
Accepted	Review Article	*Marta G. Silva, Donald P. Knowles, Monica L. Mazuz, Brian M. Cooke, and Carlos E. Suarez*	Stable transformation of Babesia bigemina and Babesia bovis using a single transfection plasmid	***Scientific Reviews***	8 : 6096 2018	Joint

Appendix

1. Generation of a transfected parasite line expressing both gfp-bsd and the tick protective antigen Bm86

We generated a plasmid transfection construct BARD-Bm86-GFP-BSD. Novel features of the transfection construct were: [i] inclusion of a MSA-1 signal peptide to direct the protein to the parasite surface, and [ii] a Histidine tag (6 Histidine residues) at the3' end of the Bm86 gene that facilitates tracking with anti His antibodies. In addition the His tag allows purification of recombinant Bm86 produced by the transfected parasites. The transfection plasmid is depicted in Figure 1.

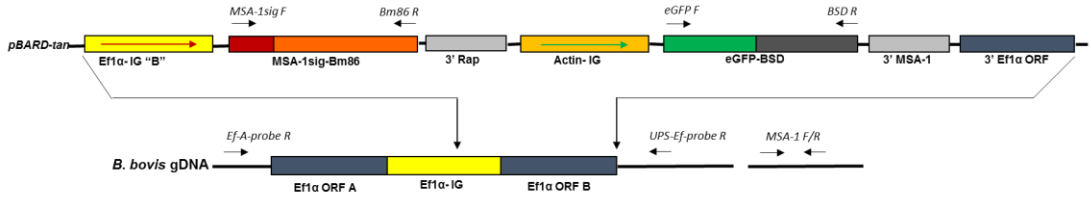

Figure 1: Schematic representation of plasmid *pBS-BARD* designed for integration in the *ef-1α* locus of *B. bovis* .

Western blot revealed that *B. bovis* parasites transfected with the pBARD plasmid, termed line Tf-183-3, are able to express a protein product recognized by anti-Bm86 antibodies (Figure 2, lane 2, anti Bm86).

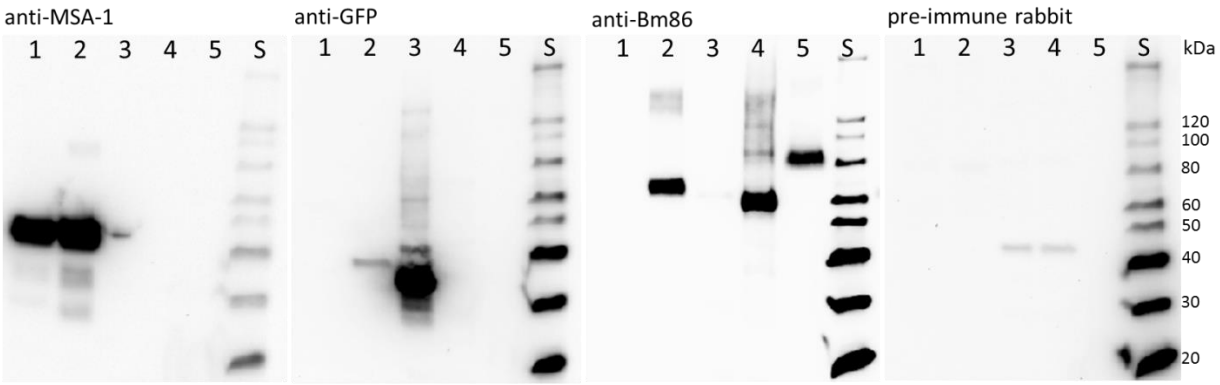

Figure 2: Immunoblot analysis of lysates generated from 1) *B. bovis* Tx, 2) B. bovis Tf-183-3, 3) recombinant eGFP-BSD, 4) recombinant Bm86, and 5) R. microplus midgut, using A) anti-MSA-1 monoclonal antibody Babb35, B) rabbit anti-GFP, C) rabbit anti-Bm86 peptides and C) pre-immune rabbit. Molecular weight shown on the right are representative of the size markers indicated by S. Furthermore, immunofluorescence analysis demonstrated targeting if the Bm86 protein to the surface of the parasites, likely as a result of the inclusion of the MSA-1 signals into the fusion Bm86 gene (Figure 3, Panels C and D, 488 and 555).

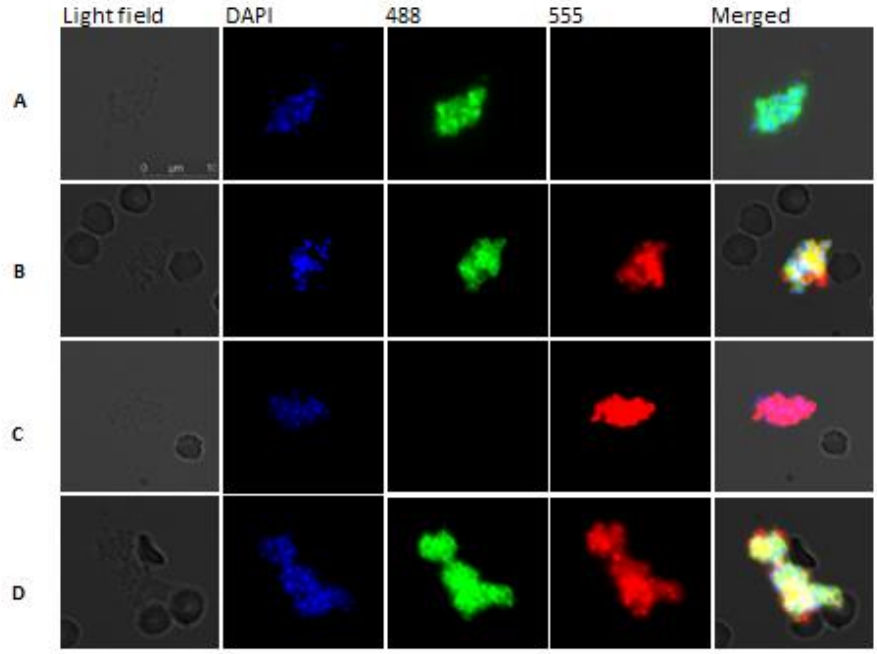

Figure 3: Surface expression of Bm86 in transfected *B. bovis* Tf-183 demonstrated by immunofluorescence analysis. **Panel A:** *B. bovis* T3Bo permeabilized free merozoites incubated with anti-MSA-1 (488) and anti-Bm86 (555) antibodies. **Panel B:** *B. bovis* Tf-183 permeabilized free merozoites, incubated with anti-GFP (488) and anti-Bm86 (555) antibodies. **Panel C:** *B. bovis* Tf-183 intact free merzoites, incubated with anti-GFP (488) and anti-Bm86 (555) antibodies. **Panel D:** *B. bovis* Tf-183 intact free merozoites, incubated with anti-MSA-1 (488) and anti-Bm86 (555) antibodies.

2. Transient and stable Transfection of *B. bigemina*

In a collaborative effort we developed transient and stable transfection systems for *Babesia bigemina* parasites and prepared a manuscript describing transient transfection and interchangeable cross-species promoter function. The future goal is obtaining transfected *B. bigemina* transfected parasites able to express the Bm86 gene as an alternative delivery system, especially in areas were *B. bigemina* vaccination alone is needed. The stable transfection of *B. bigemina* was also achieved (joint US-Israel

published manuscript). An interesting observation from these studies is that the *B .bovis* and *B. bigemina ef-1a* promoters are exchangeable among these two parasites, thus facilitating the preparation of transfection plasmids and avoiding undesired patterns of insertion of the plasmids into the genomes.

3. Confirmation of the lack of transmissibility of the *B. bovis* Israeli vaccine strain by *R. annulatus* ticks.

We also demonstrated that the *B. bovis* vaccine strain routinely used for vaccination in Israel appears to be not tick transmissible. To confirm the lack of transmission by ticks an experiment using splenectomized calf was performed.

For the acquisition feeding a 3 months splenectomized calf was infected with *R. annulatus* larvae from 0.3 gr of eggs. Fifteen days after tick infection, the calf was inoculated IV with 10 doses of *B. bovis* vaccine (contained the equivalent of 10^9 parasites). Parasitemia was first observed six days after *B. bovis* inoculation (21 days after tick infection) and reach 5% nine days pos inoculation. At this stage the animal present clinical signs of babesiosis with fever reaching 41^0C, decreased hematocrite and apatic. The animal were therefore treated with diminazene aceturate (Berenil©) to prevent development of higher parasitemia and death. Engorged females were collected during the parasitemia period (23 to 24 days after infection with the larvae). The level of survival and fecundity were similar to those obtained in engorgement of ticks in non-infected calves. The eggs mass was around 60% of the female weight and is the same obtained in routine grow of uninfected ticks.

The ovaries (after oviposition), the eggs and the larvae obtained from the engorged females were tested and found positive for *B. bovis* DNA by PCR. For the transmission feeding 2 calves of 4 months age, one splenectomized and one intact calf, were infected with larvae (from 0.4 gr of eggs) from the ticks obtained in the acquisition experiment. The *B.bovis* infected larvae (as confirmed by PCR) used for the transmission of the vaccine strain were those obtained at the peak of parasitemia (5%) at days 23 -24 after infection. After infection of calves no clinical sign of babesiosis, no fever, parasitaemia or positive PCR were observed. The results proved that the *B. bovis* vaccine strain routinely used for vaccination in Israel is not tick transmissible and thus, safer to use in field.

4. Development of a quantitative real time PCR

For follow up of the infection in vaccinated or chronically infected animals a quantitative real time PCR based on the conserved gene BBOV_III010230, designated bov230 was developed. This gene is one out of 245 putative conserved protein-coding genes scattered

across chromosomes 4 and 3and a single-copy gene with no predicted introns, encoding a protein with the predicted MW of 46,542 Da. Sequences analyses of the *bov230* among different Israeli *B. bovis* isolates revealed a high degree of conservation among the Israeli vaccine strains and isolates. Alignment of the *bov230* gene showed that the total sequence identity among the nucleic acids of the Israeli vaccine strains and field isolates ranged from 98.5 to 100% (data not shown). The sensitivity of the real-time PCR assay was determined by using dilution series of the positive control recombinant plasmid containing *bov230* gene. Approximately 1000 copies of *bov230* gene can be reliably detected in a real-time PCR (Fig. 4). Thousand copies of parasites were equivalent to a dilution of 10-7 parasites /ml of blood. The specificity of the test was shown by using other related parasites as a template. No amplification was detected when DNA from *B. bigemina, A. marginale* or *T. annulata* were used as the template.

Figure 4. Sensitivity of the *bov230* real-time PCR. Standard curve was obtained by amplification of recombinant plasmid after serial dilution. The Cq values obtained from the real-time PCR assay were plotted against numbers of plasmid DNA copies. Recombinant *plasmid* dilutions with concentrations ranging from 0.1 ng to 0.1 fg (corresponding to 10^7–10^3 copies of target DNA).

The qPCR established in this study is of high sensitivity and specificity, for quantitative detection of *B. bovis* in cattle and can provide a useful tool for the follow up of the vaccination trials programed to the next year of this project.

5. Persistent infection of transfected parasites with continuous production of Bm86 antibodies in splenectomized cattle

The transfected *B. bovis* parasite line *B. bovis* Tf-183, expressing Bm86 was generated in the US lab and transferred to Israel. Two splenectomized 4 months old calves were infected I.V.

with $2X10^8$ frozen live *B. bovis* Tf-183 parasites in order to confirm parasite viability, amplify the parasite line, and to generate fresh frozen stabilates. Follow up of the animals after infection included fever, PCV and parasitemia. Blood were taken once a week for performing serology to detect antibodies against the parasite and Bm86, and to extract DNA for PCR analysis. The PCRs were aimed at detecting DNA of the parasite and the Bm86 gene present in transfected parasites. A third splenectomized calve (number 97) was infected with 50ml blood from calve number 89 when parasitemia was less then 0.005% and the PCV was 24. Follow up data of the clinical signs after infection of splenectomized animals is described in Table 1. A transient period of fever and decrease of PCV were observed in the period of visible parasitemia. None of the infected animals required treatment after experimental infection.

Calf number	**Infected with**	**Fever**			**Parasite**		**PCV**		**Microscopic fluorescence in direct blood smears**
		Begin at (d.p.i.)	**Days**	**Max [Cº]**	**Days of visible PPE**	**Max %PPE**	**Min**	**day**	
89	*B. bovis* Tf-183 , 1.5ml I.V. frozen live $2X10^8$ parasites	10	6	40.9	10-17	0.1%	23	17	no
96	50 ml blood from calf 89 with PPE<0.005%	4	6	40.8	4-10	0.5%	19	17	no
97	*B. bovis* Tf-183 , 1.5ml I.V. frozen live $2X10^8$ parasites	10	5	41.1	10-15	0.5%	24	14	yes

Table 1. Clinical follow up data of splenectomized calves infected with transfected *B. bovis* Tf-183 parasites

Persistent infection was observed during at least six month by PCR examination (data not shown). Positive PCR for the Bm86 gene were also observed in the period of higher parasitemia (two weeks after infection) after it, positive PCR to Bm86 was observed only in

calf number 97 for at least 67 days. Serology against *Babesia* was positive during all followed period, with antibodies titers by IFAT varying from 1:64 to 1:1024.

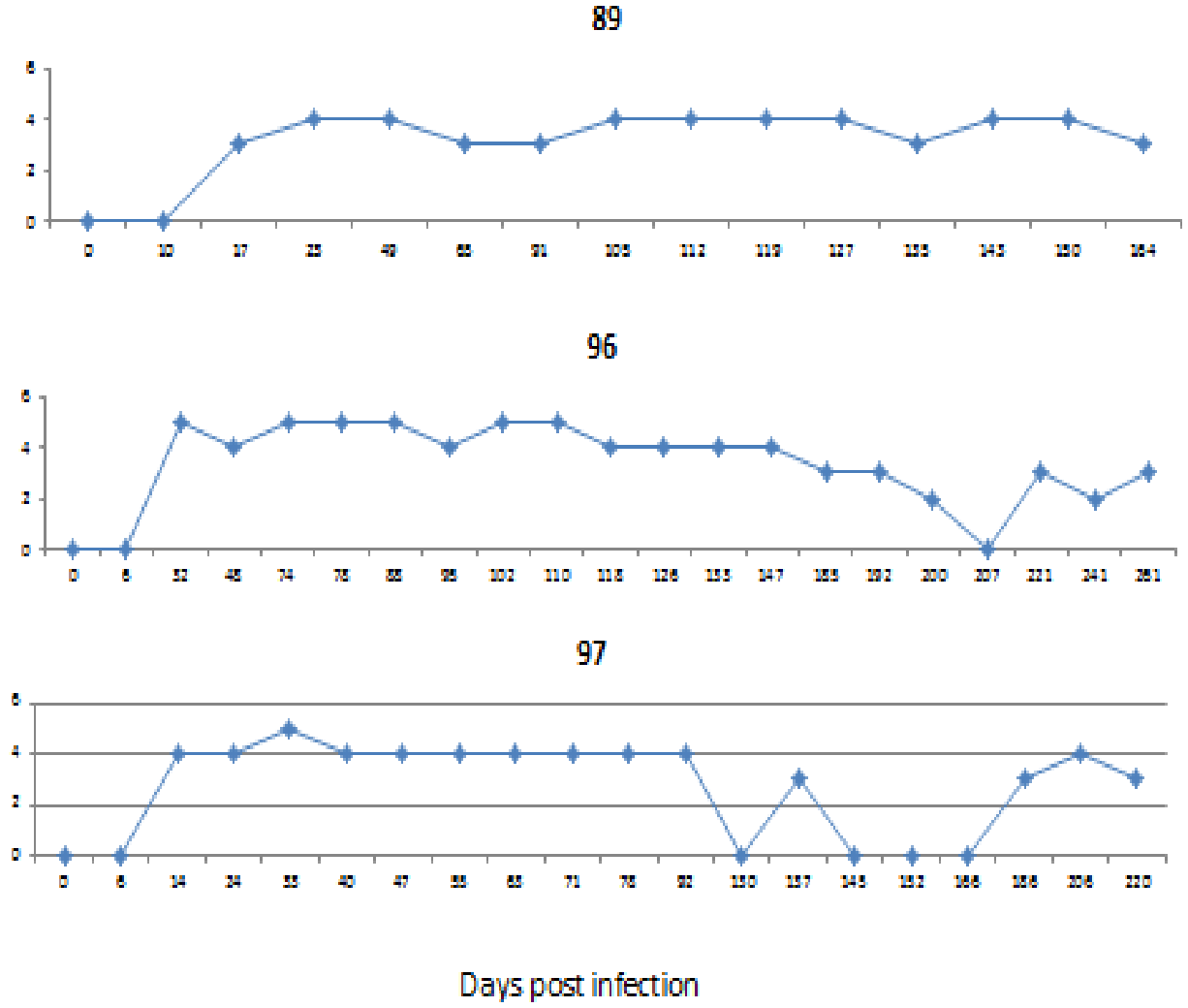

Figure 5. Serology for the detection of *B. bovis* infection (IFAT) performed on splenectomized calves after infection with *B. bovis* transfected parasites. The titers are shown in log 4.

Vaccination with the *B. bovis* Tf-183 parasites leads also to production of specific antibodies against this tick antigen. As observed in the ELISA test performed, an increase in the index value was observed two weeks after infection and a constant humoral immune response remain during all the observed period (Figure 6).

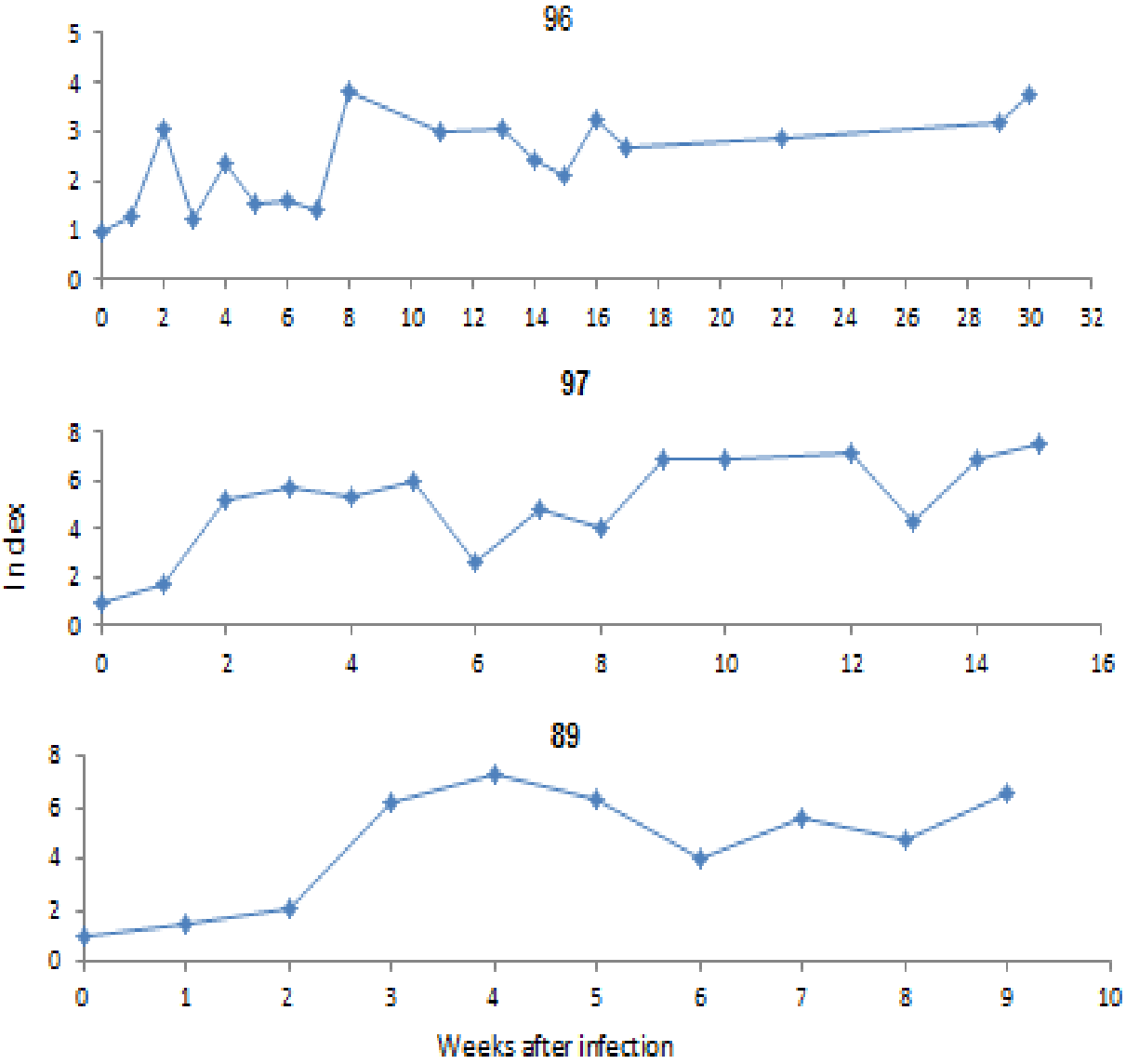

Figure 6: Index values corresponding to the production of antibodies against Bm86 in calves in splenectomized calves numbered 96, 97 and 89, as detected by ELISA. The index value was calculated by dividing the OD observed in ELISA tests in the day of sampling by the OD observed before vaccination.

Two splenectomized animals were submitted to post mortem examination 9 months after infection. No macro-pathology was observed in both animals. Microscopic and molecular examination of the organs of the persistent infected splenectomized calves, showed sequestration of parasites in different organs, including the brain (Fig.7).

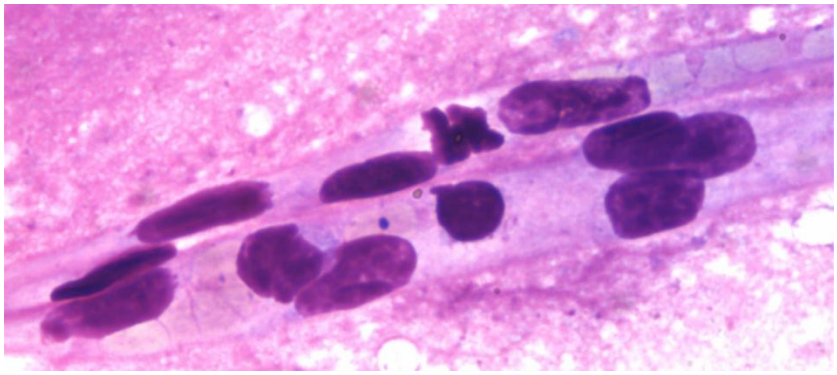

Figure 7. Brain smear of the calf number 96 showing infected erythrocytes inside of the brain capillaries

PCR examination of the organs showed positive results for *Babesia bovis* and for Bm86 in diverse organs as decribed in table 3. The persistent parasitemia of transfected parasites were observed in both calves. The distribution of the parasites in several organs were presented in Table 2. **Table 2.** Detection of *B. bovis* and Bm86 DNA performed by PCR of different organs of the splenectomized infected calves number 96 and 97.

	B. bovis-specific PCR amplicons		Bm86-specfic PCR amplicons	
	96	97	96	97
Brain	+	+	+	+
Lungs	+	+	+	+
Spinal cord	+	+	+	+
kidney	+	+	-	+
liver	+	+	+	-
heart	+	+	-	+

The presence of Bm86 DNA could not be observed in all organs were *B. bovis* DNA was amplified. However, this could be due to the different sensitivities of the PCRs used in these experiments, since we used a nested PCR for the detection of *B. bovis*, but not for Bm86.

6. Partial protection against ticks

Splenectomized calves vaccinated with *B. bovis* transfected parasites expressing Bm86 were challenged with *R. annulatus* ticks in order to check for protective immune responses Calves number 96 and 97 were challenged with non-infected *R. annulatus* larvae 1 and 3 months after infection respectively. Parameters recorded included: individual tick weight, egg laying capacity, egg weight/female weight and eggs fertility. Statistical analysis (T-test) was performed to determine significance. As observed in table 3. The total number of dropped tick had been reduced (2587 total ticks in the control calf 99 compared to 690 and 624 in vaccinated calves number 96 and 97 respectively). Although the reduction in the number of dropped ticks there was no reduction in other parameters such fertility and eggs laying capacity.

Table 3. Biological parameter of detached *R. annulatus* from vaccinated splenectomized calves and control calf. SEM= standard error of mean.

	T183 infected Splenectomized calves		Control	t-value
Calf ID	96	97	99	
Total	690	624	2587	
Individual weight (mean, g)	0.26	0.26	0.27	0.249
SEM	0.05	0.03	0.04	
Eggs laying capacity	0.13	0.14	0.14	0.449
SEM	0.03	0.02	0.03	
eggs weight/female weight	0.49	0.53	0.49	0.964

SEM	0.06	0.03	0.06	
Eggs fertility	4.31	4.32	4.30	0.931
SEM	0.68	0.73	0.64	

7. Safe vaccination of intact calves against *B. bovis* using transfected and non-transfected parasites

Nine intact calves aging 4-9 months were vaccinated to determine whether cattle vaccinated with the *B. bovis* Tf-183 transfected strain of *B. bovis* were protected against challenge with a virulent strain of *B. bovis.* The calves were separated in three groups. Group 1 received $5X10^6$ frozen live *B. bovis* Tf-183parasites, group 2 received $5X10^6$ frozen live *non transfected* T2B *B.bovis* parasites, whereas animals in group 3 were not vaccinated and kept as control. Follow up of after vaccination was performed (Table 4). After vaccination transient fever, slight decrease in the PCV and low parasitemia was observed in all calves infected with non-transfected T2B *B.bovis* parasites. In calves vaccinated with the *B. bovis* Tf-183 parasites, clinical response with low parasitemia was observed only in calf number 105. The two remaining calves in group 1 had no clinical signs of infection and parasites were not detected by microscopic observation of Giemsa stained slides after vaccination. Therefore animals 103 and 107 were revaccinated with a second dose of the same vaccine. After revaccination both calves had increase in fever, decrease in the PCV and low parasitemia (Table 5).

Vaccination with the *B. bovis* Tf-183 parasites did not result in production of specific antibodies against this tick antigen. As observed in the ELISA test performed, no increase in the index value was observed two weeks after infection and further one. **(Figure 7).**

Table 4. Clinical follow up of calves after vaccination

Calf number	Age [months]	Injected strain	Incubation period / Days until first fever	Fever		Parasite			PCV	
				Days	Max [C°]	Incubation period [Days]	Days of visible PPE	Max	Time 0 / Max	
103	4.5	T-183	-	1	39.7	-	-	-	34	
105	4.5	T-183	13	3	40.1	14	3	+---	36	
107	2.5	T-183	-	2	39.7	-	-	-	35	
104	4.5	B-TBO	9	4	39.9	9	2	+--	36	
106	4.5	B-TBO	10	4	39.9	10	2	+--	33	
108	2.5	B-TBO	12	1	39.6	12	1	+--	33	
100	6.5	Control	-	0		-	-	-	38	
101	4.5	Control	-	0		-	-	-	36	
110	2.5	Control	-	0		-	-	-	35	

Table 5. Clinical follow up of calves after revaccination of calves 103 and 107

Calf number	**Fever**			**Parasites**	
	Days until first fever	**Days**	**Max [C°]**	**First observed PPE**	**Days of visib**
103	9	3	40.1	9	2
107	11	3	40.3	11	1

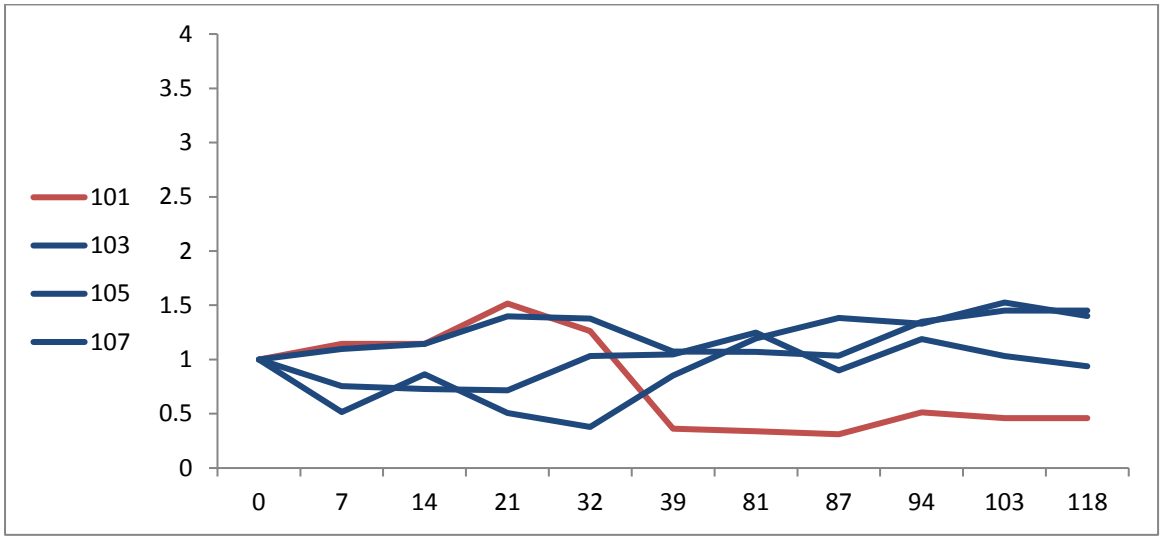

Figure7: Index values corresponding to the production of antibodies against Bm86 by ELISA, in calves vaccinated with T-183 transfected *B. bovis* parasites (103,105 and 107) and in a control calve number 101. The index value was calculated by dividing the OD observed in ELISA tests in the day of sampling by the OD observed before vaccination.

8. Ticks infestation of vaccinated animals

Three animals, one from each group of vaccinated with T-183, vaccinated with T2bo and an uninfected calves, were infected with larvae as previous described above. No effect on the parameters recorded included: individual tick weight, egg laying capacity, egg weight/female weight and eggs fertility were observed between the infected animals. Interesting although the same number of larvae were applied as in the first experiment, significant lower number of ticks were obtained in this experiment. This can be due to the different grooming behavior of the animals or due the difference between batches of ticks. Unfortunately no effect against ticks were observed in the animals vaccinated with transfected parasites containing the Bm86 antigen. The lack of effect should be better understand and further experiments using higher doses of parasites in the transfected vaccine should be considered.

Table 6. Biological parameter of detached *R. annulatus* from vaccinated splenectomized calves and control calf. SEM= standard error of mean.

Calf ID	101	104	105
Total	294	279	257
Individual weight (mean, g)	7.24	6.94	7.27
SEM	1.31	1.7	1.34
Eggs laying capacity	0.113	0.118	0.127
SEM	0.03	0.02	0.015
eggs weight/female weight	0.453	0.474	0.498
SEM	0.09	0.06	0.03
Eggs fertility	82.6	83.2	82.6
SEM	10.04	9.64	10.32

9. Challenge of vaccinated calves and conclusions

Eight animals, six vaccinated calves (3 with *B. bovis* T2bo strain and 3 with *B. bovis* transfected T-183 parasites) and two non-vaccinated animals were challenged 5 months after vaccination. The challenge was performed with1,5 ml blood containing *B. bovis* Katcha virulent strain originated from an infected cow with 4,5% parasitemia. Clinical signs of babesiosis with high fever and decrease in the PCV and countable parasitemia were observed in all groups. All the groups recover from the challenge without treatment. Although no statistical significant differences were observed between groups the general clinical situation of thee group vaccinated with T2bo strain was better comparing with the two other groups, based on fewer days of fever and lower temperature compared to the other groups. However, as all animals recovered from challenge without treatment and no significant differences were observed between groups, the efficiency of the transfected parasites as potential future vaccine against virulent Babesia remains to be determined experimentally.

Overall the data suggests strong trends into the desired outcomes, the vaccination trial should be re-performed using higher doses of parasites that could induce both immune response against the Bm86 antigen as observed in the splenectomized calves and effective protection against virulent strains. In addition, the data suggest that the next set of vaccination/challenge experiments should be performed on older animals, which are more sensitive to Babesia infection, in order to acquire more conclusive data on the protective effects of the vaccination against virulent strains in field.

Despite the failures in the testing using intact animals, substantial progress towards the development of a dual *Babesia*-tick vaccine was achieved in this project. Important and original developments include producing exogenous antigens in transfected Babesia, the targeting of the Bm86 antigens in the surface of the parasites and the elicitation of Bm86 antibodies in the splenectomized vaccinated animals. However, our animal trials were limited in size and definitive data on the effectivity of the vaccine remains to be established using larger numbers of animals, with diverse ages. In addition, it is possible that Bm86 is not a highly effective anti-tick vaccine antigen, and the approach can be attempted using other alternative protective tick antigens.

Overall, the data supports the feasibility of developing the proposed approach of expressing exogenous tick antigens in transfected Babesia, since we were able to obtain consistent antibody responses in splenectomized animals. It is expected that the US and Israel labs will continuing the collaboration towards improving and perfecting the new vaccine approach generously financed with the BARD grant.

Table 7. Clinical follow up of calves after challenge with virulent *B. bovis* parasites

Calf number	Age [months] at challenge	Injected strain	Incubation period / Days until first fever	Fever		Parasite		
				Days	Max [C°]	Incubation period [Days]	Days of visible PPE	Max PPE
103	9.5	T-183	10	6	41.7	10	6	0.5
105	9.5	T-183	14	6	41.7	17	1	0.1
107	7.5	T-183	11	7	41.5	11	1	0.1
104	9.5	B-TBO	10	3	40.2	10	4	0.1
106	9.5	B-TBO	10	3	41.1	10	3	0.1
108	9.5	B-TBO	9	5	40.2	9	5	0.1
101	9.5	Control	9	6	41.1	9	6	0.5
110	7.5	Control	11	5	41	13	2	0.1